AD_____

Award Number: W81XWH-04-1-0532

TITLE: Defining the Molecular Actions of Dietary Fatty acids in Breast Cancer: Selective Modulation of Peroxisome Proliferator-Activated Receptor Gamma

PRINCIPAL INVESTIGATOR: Clinton D. Allred, Ph.D.

CONTRACTING ORGANIZATION: Kentucky University
Lexington, KY 40506-0057

REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual (9 Apr 2004 - 8 Apr 2005)

4. TITLE AND SUBTITLE
Defining the Molecular Actions of Dietary Fatty acids in Breast Cancer: Selective Modulation of Peroxisome Proliferator-Activated Receptor Gamma

5. FUNDING NUMBERS
W81XWH-04-1-0532

6. AUTHOR(S)
Clinton D. Allred, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Kentucky University
Lexington, KY 40506-0057

E-Mail: c.allred@uky.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Stark differences in the actions of lenoleic acid (LAA), an omega-6 fatty acid, and eicosapentaenoic acid (EPA), an omega-3 fatty acid, on breast cancer tumors have been described. We propose that transactivation of peroxisome proliferators-activated receptor gamma (PPARγ) mediates the physiological effects of different dietary fatty acids on breast cancer. PPARγ plays a role in the development and progression of breast cancer tumors. We have demonstrated that individual ligands of PPARγ can selectively activate PPARγ in three different ways. Selective activation of PPARγ by a single ligand occurs between tissue types and between individual breast cancer cell lines. Also, unique ligands selectively activate PPARγ within a single cell type. We propose that fatty acids will elicit their effects on breast cancer cells in a similar manner. The objectives of forth coming studies will be to assess the necessity of transactivation of PPARγ by LAA and EPA in mediating the effects of fatty acids on breast cancer cells and to determine what effect this selective activation has on gene transcription. We hypothesize that LAA and EPA can selectively regulate gene expression profiles that favor either cellular differentiation or proliferation and that these effects are dependent on transactivation of PPARγ.

14. SUBJECT TERMS
Breast cancer, dietary fatty acids, PPPAR, nuclear receptor, molecular

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....10

Reportable Outcomes.....10

Conclusions.....10

References.....11

Appendices.....13

Introduction

Breast cancer remains one of the leading forms of cancer in American women. One in every eight women in the United States will be diagnosed with breast cancer, a 5-fold higher rate than that observed in women living in Japan and China. Studies examining women that emigrate from Asian countries to the United States have found similar breast cancer risk as American women within 40 years suggesting that genetics alone can not account for differences in breast cancer incidence between these populations (*1*). A number of environmental aspects are being explored to elucidate factors that might influence breast cancer risk. Though controversial, it has been estimated that diet influences the development of up to 50% of all breast cancer cases in American women (*2*). A growing body of literature indicates that the type of dietary fat consumed (diets high in omega-3 fatty acids versus diets high in omega-6 fatty acids) influences breast cancer (*3, 4*) suggesting that consumption of specific fatty acids may impact breast cancer differently. Animal studies, have also provided convincing evidence of a correlation between types of fats ingested and mammary tumor development and growth (*5-8*). The focus of this proposal is to define the molecular link between specific fatty acids and the progression of breast cancer. We are exploring the possibility that fatty acids may elicit their effects in breast cancer cells by acting as ligands of the peroxisome proliferator-activated receptor gamma (PPARγ). Our laboratory has previously demonstrated that individual fatty acids can activate a PPAR-response element (PPRE), but whether this effect was the direct result of PPARγ activation has been left unexplored (*9*). Evidence suggests PPARγ is involved in the initiation (*10, 11*) and progression (*12-14*) stages of breast cancer. The objective of this proposal is to determine the mechanism of action that individual fatty acids use to either positively (increase cellular differentiation and/or decrease cellular proliferation) or negatively (increase cellular proliferation and/or tumor metastases) impact breast cancer cells. We propose that PPARγ is the molecular target responsible for the physiological effects of different dietary fatty acids on breast cancer.

Body

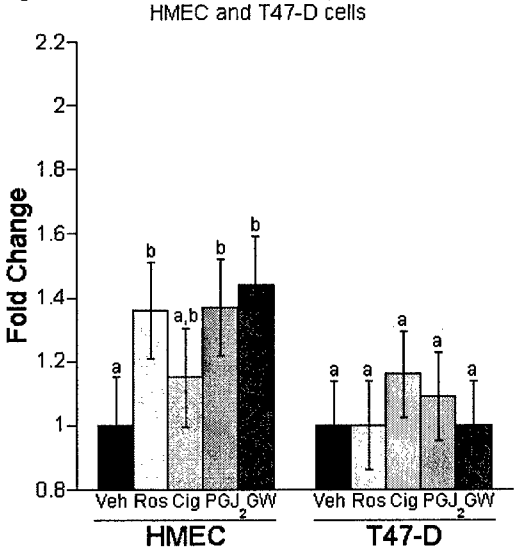
The orphan nuclear receptor, PPARγ is one of three in a family of receptors (PPAR α, β, and γ) (*15-17*). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (*18-22*). Several ligands of PPARγ have been identified including 15-deoxy-Δ12,14-prostaglandin J_2 (PGJ_2), linoleic acid (LAA), lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone (Cig) and rosiglitazone (Ros) (*9, 23-26*). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXRα), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (*22, 27*).

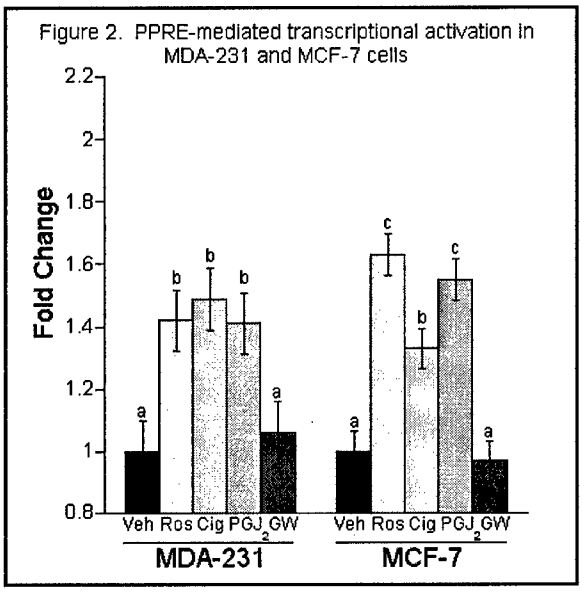
To begin to explore the role that individual fatty acids might play in the progression of breast cancer we wanted to first demonstrate that individual ligands of PPARγ could selectively modulate the receptor. The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (*28-30*). It has been proposed that individual ligands may be able to act as selective PPARγ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (*31*). We previously demonstrated that individual fatty acids can selectively activate a PPRE-reporter assay in estrogen-dependent breast cancer (MCF-7) cells (*9*). However, left unexplored was whether individual ligands could elicit unique responses between cell lines of a single cancer type as well as across cancers of different tissue origin.

To address these fundamental questions we have conducted a series of experiments that tested the ability of individual PPARγ ligands to selectively activate PPARγ. To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. The data from these studies has been collected, analyzed, and the resulting manuscript was recently accepted for publication in the May issue of Molecular and Cellular Endocrinology (see appendix).

In these studies, we first tested the ability of several different PPARγ ligands to activate the PPRE-reporter in either normal mammary epithelial (HMEC), estrogen-dependent breast cancer cells (T47-D and MCF-7), or estrogen-independent breast cancer cells (MDA-MB-231). Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were treated with either vehicle control or PPARγ ligands for 18 hours. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and PGJ_2 both significantly increased reporter activity over control (Fig. 1). Interestingly, GW, a known antagonist of PPARγ, also significantly stimulated reporter activity in HMECs. GW treatment did not change reporter activity compared to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1). In MDA-MB-231 cells Ros, Cig, and PGJ_2 all significantly enhanced PPARγ activation over control, while these same three treatments also increased reporter activation in MCF-7 cells when compared to control (Fig. 2). Both Ros and PGJ_2 treatments resulted in significantly higher activity than Cig in MCF-7 cells.

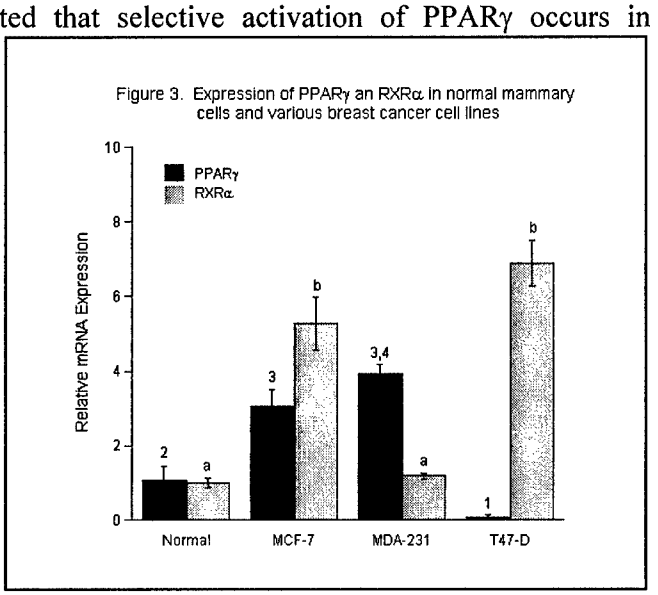
Figure 1. PPRE-mediated transcriptional activation in HMEC and T47-D cells





After a breast tumor has formed, PPARγ transactivation appears to have multiple effects. In vitro, treatment of breast cancer cells with troglitazone results in lipid accumulation, changes in gene expression associated with cellular differentiation, reduction in growth rate and clonogenic capacity (*11*). Others have observed that distinct PPARγ ligands induce apoptosis (*13*). Conversely, a recent report by Saez *et al.* found that when mice expressing a constitutively active form of PPARγ in the mammary gland were crossed with mice prone to mammary gland cancer, bigenic animals develop tumors that express higher levels of markers of malignancy (*32*). The authors conclude that once an initiating event takes place, increased PPARγ signaling serves as a tumor promoter in the mammary gland of these experimental animals. Collectively, these data suggest that the physiological consequence of PPARγ activation is dependent on many factors including the stage of development of the specific breast cancer cell. Our demonstration that individual PPARγ ligands distinctively modulate PPRE reporter activity in breast cancer cell lines differently has implications for breast cancer treatment. Specifically, T47-D cells were fairly unresponsive to any of the three PPARγ agonists tested, whereas, Ros, Cig, and PGJ_2 significantly increased reporter activity in MCF-7 and MDA-MB-231 cells. It can be concluded that individual breast cancer cell types are likely to respond to PPARγ ligands in unique physiological ways and our data suggests that, in part, variant cellular responses are the result of selective PPARγ transactivation. Furthermore, we report that GW, a known PPARγ antagonist in adipocytes (*33, 34*) and other cell lines, significantly increased reporter activity in HMECs. These findings are significant because they suggest that an individual compound can function as a PPARγ antagonist in one tissue and as an agonist in other tissues. It is possible that the agonist activity of GW is specific to normal epithelial cells and that changes occur during cancer cell formation that results in the loss of this responsiveness. It is also possible that the actions of GW are mammary specific. Further investigation is necessary to explore these possibilities.

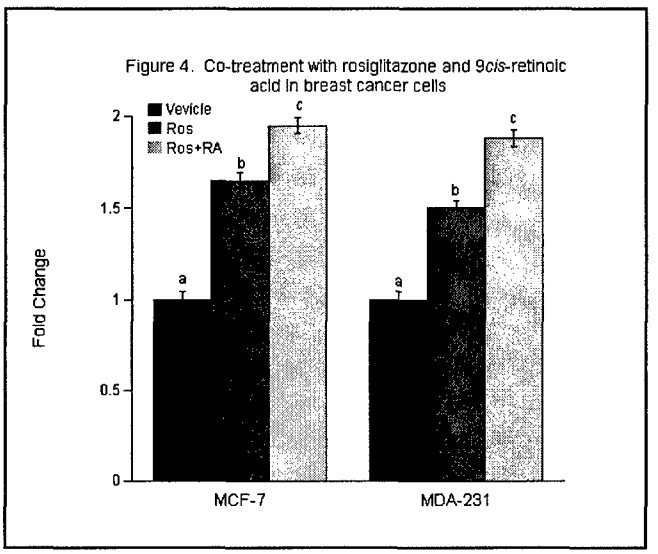
Data from these experiments demonstrated that selective activation of PPARγ occurs in multiple ways. Distinct ligands selectively activate PPARγ dependent on the tissue type from which the cell line was derived (data not shown, see MCE manuscript for full description). SPARM activity was also observed between different cell lines of the same tissue origin. Specifically, normal mammry cells and breast cancer cells responded differently to individual ligands and differences were observed between unique breast cancer cell lines. Also, individual ligands selectively activated the PPRE reporter within single breast cancer cell lines.



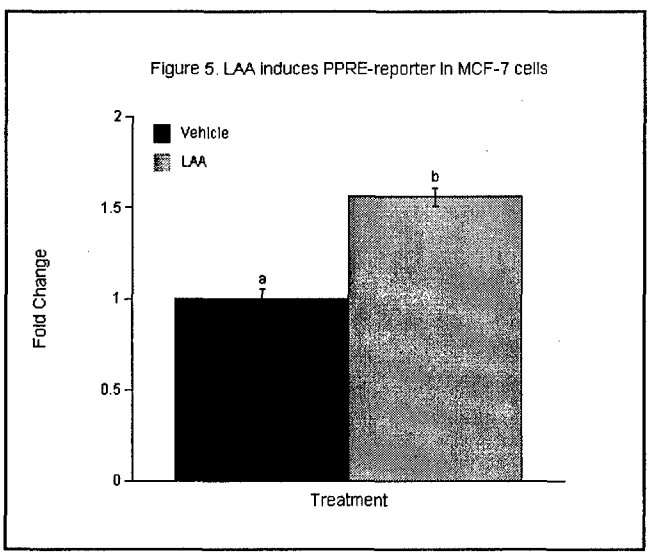
Next we wanted to determine if mRNA levels of PPARγ and its heterodimic partner,

RXRα, in cells were predictive of how the breast tumor cells would respond to PPARγ ligands. To do this, mRNA concentrations of PPARγ1 and RXRα were measured in all four cells lines (HMEC, T47-D, MDA-MB-231, and MCF-7) used previously in the transfection assays in the absence of ligand treatment. Data is presented as fold change in expression compared to the HMECs. T47-D cells had significantly lower levels of PPARγ than all of the cell lines (Fig. 3). However, these cells had the highest expression of RXRα among all cell lines. MCF-7 cells express significantly higher levels of PPARγ expression than the HMECs or T47-Ds and higher RXRα than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPARγ mRNA levels similar to MCF-7 cells, but had lower RXRα expression.

We then chose two breast cancer cell lines to determine if unique patterns of expression of PPARγ and RXRα would be predictive of how the cells responded to co-treatment with Ros (a PPARγ agonist) and 9*cis*-retinoic acid (a RXRα agonist). MDA-MB-231 cells express higher levels of PPARγ relative to RXRα and would therefore, not be expected to have increased reporter activity with co-treatment (Ros + 9*cis*-retinoic acid) compared to Ros alone. Conversely, MCF-7 cells having higher expression of RXRα relative to PPARγ would demonstrate enhanced reporter activity when treated with both ligands compared to Ros alone. MCF-7 cells had enhanced activation of the PPRE reporter with co-treatment over Ros treatment alone (Fig. 4). The MCF-7 cell line supports the possibility that relative expression levels may be predictive in identifying cells that will more readily respond to co-treatment with both ligands. However, MDA-MB-231 cells did not support this hypothesis. Co-treatment with both ligands had a significantly higher effect on reporter activity when compared to single treatment with Ros even though relative PPARγ expression was higher than RXRα expression in these cells. These data suggest that while this approach may work for certain cancer cell types, receptor expression alone may not be predictive for how cells will respond to co-treatment.



Data discussed thus far has identified the possibility that unique PPARγ ligands can function as SPARMs in several ways that may have a significant impact on breast cancer development and progression. Furthermore, these data provide in principal the potential mechanism by which fatty acids may function uniquely in breast cancer cells. Beyond collecting the previously discussed data we have spent the first year of the funding schedule developing methodology to effectively deliver fatty acids into the cells. Because of the number of different outcomes that will be analyzed in this grant we wanted to develop a protocol for fatty acid delivery that would provide us a reliable methodology. In the last twelve months, we



attempted several different delivery methods using the PPRE reporter assay as a means to measure the efficacy of each protocol. Early on, we were unsuccessful at getting agonistic responses with several of our fatty acid protocols. However, we have now developed a protocol that has provided us a mechanism of fatty acid delivery that results in measurable responses in the PPRE reporter assay (Fig. 5). Furthermore, this methodology is reproducible and reliable for use in a number of different assays. The details of the fatty acid protocol are listed in the methodology below. We anticipate that this form of fatty acid delivery will be used throughout the remainder of the experiments funded by this grant.

Methods:

Cell Culture: HMEC, MDA-MB-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscript.

Transfection Assays: Cells were transiently transfected with 5μg of PPRE reporter plasmid per 12 well plate. Cells were transfected with ESCORT transfection reagent for four hours. Cells were subsequently treated with either 10μM Ros, 10μM Cig, 1μM PGJ_2, 1μM GW, or 150μM LAA for 18 hrs. In all cases, PPARγ ligand concentrations for each compound used were those shown to be maximally effective following dose reponse studies. Proper vehicle controls including ethanol, DMSO, and methyl acetate were run for each treatment group. Following treatment, cells were lysed in 50μl passive lysis buffer and treated according to manufacturer's instructions (Promega dual luciferase assay kit). Luminometry was performed on a Berthold Lumat 9507 and data calculated as raw Luciferase units (RLU's) divided by raw Renilla units. Mean fold induction was obtained by dividing the RLU data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of 6 in 3 separate experiments.

RT-PCR: Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems and used according to manufacturers instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to asses PPARγ and RXRα mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5' nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a C_t cycle method. At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number (C_t), which is inversely correlated to the abundance of the initial message level. C_t measures the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH, again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target C_t value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct$ represents ΔCt, target sample minus ΔCt, calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

Fatty Acid Preparation: Fatty acids were purchased in pure fatty acid form and then dissolved in hexane to create a fatty acid stock solution. This stock solution was maintained under nitrogen gas at all times and fresh fatty acid preparations were made before every experiment. Appropriate volumes of the stock solution were then combined with calculated volumes of 6N NaOH to form fatty acid salt complexes. The preparations were then dried under nitrogen gas until no fluid remained. The fatty acid salt was then dissolved in cell culture media containing 10% fetal bovine serum (FBS). It has been reported that the availability of free fatty acids in the body is dependent on the presence of

albumin and therefore depends on albumin concentration (*35*). As a result, the chosen FBS conditions were necessary to form fatty acid/albumin complexes. Once the fatty acid was completely dissolved in the media, hydrochloric acid was used to balance the pH and the media was filter sterilized through a 0.2μm syringe filter.

Key Research Accomplishments

- We have demonstrated that individual PPARγ ligands can selectively activate the receptor in cancer cell lines derived from different tissues.
- We have shown that selective modulation of PPARγ occurs between normal mammary epithelial cells as well as different breast cancer cell lines when the cells are treated with PPARγ ligands. These data suggest that breast tumors in individual patients may respond to PPARγ differently.
- We have shown that individual PPARγ ligands can selectively modulate the receptor within a single cell line.
- We have demonstrated that distinct expression patterns of RXRα and PPARγ mRNA in tumor cells may be predictive of how they will respond to PPARγ ligand treatment, but further investigation is necessary to better define this approach.
- We have now developed a methodology for delivering fatty acids into cells that is reproducible and reliable for use in all future experimentation.

Reportable Outcomes

- Initial data resulting from this funding has led to the preparation and acceptance of a peer reviewed manuscript to be published in Molecular and Cellular Endocrinology in May of 2005.
- Data supported by this grant was presented in a poster format at the Twenty Third Annual University of Kentucky Symposium in Reproductive Sciences in May of 2004.
- Collected data has led to an invited oral presentation at the Twenty Fourth Annual University of Kentucky Symposium in Reproductive Sciences in May of 2005.
- The most recent data associated with this grant will be presented at the 2005 Era of Hope meeting.
- Through studies and collaborations associated with this grant I have become active in the Reproductive Sciences Training Program at the University of Kentucky. This is a federally funded program and prior to the 2004/2005 academic year I was selected to act as seminar coordinate for this program.

Conclusions

PPARγ is highly expressed in breast cancer tumors and treatment of these cells with known PPAR agonists *in vitro* have been shown to suppress tumor cell growth. This has led to the possibility that PPARγ may be utilized as a therapeutic target in the treatment and prevention of breast cancer. Thus far, we have demonstrated that PPARγ ligands, have distinct activities within a cell type, between tumor cells derived from the same tissue, and across distinct tissues. These general principles are the mechanism by which we hypothesize that individual fatty acids influence breast cancer development and progression. With reliable methodology for delivering the free fatty acids to the breast cancer cell lines in place, we are poised to shed new light on the molecular pathway by which fatty acids function in these cells. The end result of these studies will be a stepping stone toward developing dietary recommendations for fatty acid consumption for patients with breast cancer as well as those at high risk of developing the disease.

Literature Cited

1. R. G. Ziegler *et al.*, *J Natl Cancer Inst* **85**, 1819-27 (1993).
2. R. Doll, *Cancer Res* **52**, 2024s-2029s (Apr 1, 1992).
3. J. R. Hebert, A. Rosen, *Cancer Detect Prev* **20**, 234-44 (1996).
4. L. Kaizer, N. F. Boyd, V. Kriukov, D. Tritchler, *Nutr Cancer* **12**, 61-8 (1989).
5. L. A. Cohen, J. Y. Chen-Backlund, D. W. Sepkovic, S. Sugie, *Lipids* **28**, 449-56 (1993).
6. J. M. Connolly, X. H. Liu, D. P. Rose, *Nutr Cancer* **29**, 48-54 (1997).
7. A. J. Parkinson *et al.*, *Am J Clin Nutr* **59**, 384-8 (1994).
8. D. P. Rose, *Am J Clin Nutr* **66**, 1513S-1522S (1997).
9. S. R. Thoennes, P. L. Tate, T. M. Price, M. W. Kilgore, *Mol Cell Endocrinol* **160**, 67-73 (2000).
10. Z. Zou *et al.*, *Science* **263**, 526-9 (1994).
11. E. Mueller *et al.*, *Mol Cell* **1**, 465-70 (1998).
12. C. E. Clay *et al.*, *Carcinogenesis* **20**, 1905-11 (1999).
13. E. Elstner *et al.*, *Proc Natl Acad Sci U S A* **95**, 8806-11 (1998).
14. S. K. Kachhap, P. Dange, S. Nath Ghosh, *Cancer Lett* **154**, 115-20 (Jun 30, 2000).
15. C. Dreyer *et al.*, *Cell* **68**, 879-87 (Mar 6, 1992).
16. I. Issemann, S. Green, *Nature* **347**, 645-50 (1990).
17. S. A. Kliewer *et al.*, *Proc Natl Acad Sci U S A* **91**, 7355-9 (1994).
18. O. Braissant, F. Foufelle, C. Scotto, M. Dauca, W. Wahli, *Endocrinology* **137**, 354-66 (1996).
19. M. W. Kilgore, P. L. Tate, S. Rai, E. Sengoku, T. M. Price, *Mol Cell Endocrinol* **129**, 229-35 (1997).
20. T. Lemberger *et al.*, *Ann N Y Acad Sci* **804**, 231-51 (Dec 27, 1996).
21. L. Nagy, P. Tontonoz, J. G. Alvarez, H. Chen, R. M. Evans, *Cell* **93**, 229-40 (1998).
22. P. Tontonoz, E. Hu, R. A. Graves, A. I. Budavari, B. M. Spiegelman, *Genes Dev* **8**, 1224-34 (1994).
23. B. M. Forman *et al.*, *Cell* **83**, 803-12 (1995).
24. S. A. Kliewer *et al.*, *Proc Natl Acad Sci U S A* **94**, 4318-23 (1997).
25. T. M. Larsen, S. Toubro, A. Astrup, *Int J Obes Relat Metab Disord* **27**, 147-61 (Feb, 2003).
26. T. M. McIntyre *et al.*, *Proc Natl Acad Sci U S A* **100**, 131-6 (Jan 7, 2003).
27. S. A. Kliewer, K. Umesono, D. J. Noonan, R. A. Heyman, R. M. Evans, *Nature* **358**, 771-4 (1992).
28. B. Fisher *et al.*, *J Natl Cancer Inst* **90**, 1371-88 (Sep 16, 1998).
29. V. C. Jordan, M. Morrow, *Endocr Rev* **20**, 253-78 (Jun, 1999).
30. A. S. Levenson, V. C. Jordan, *Eur J Cancer* **35**, 1974-85 (Dec, 1999).
31. M. B. Sporn, N. Suh, D. J. Mangelsdorf, *Trends Mol Med* **7**, 395-400. (2001).
32. E. Saez *et al.*, *Genes Dev* **18**, 528-40 (Mar 1, 2004).
33. L. M. Leesnitzer *et al.*, *Biochemistry* **41**, 6640-50 (May 28, 2002).
34. K. Starkey *et al.*, *J Clin Endocrinol Metab* **88**, 55-9 (Jan, 2003).
35. A. A. Spector, *Ann N Y Acad Sci* **149**, 768-83 (Nov 21, 1968).

Abbreviations

- PPARγ- peroxisome proliferator-activated receptor gamma
- PPRE- PPARγ response element
- RXRα- retinoid X receptor alpha
- TZD- thiazolidinedione
- Ros- rosiglitazone
- Cig- ciglitazone
- PGJ_2- 15-deoxy-delta 12,14-PGJ_2
- GW- GW9662
- LAA- lenoleic acid
- RLU- renilla units
- rt-PCR- real-time polymerase chain reaction
- C_t- cycle number

ELSEVIER

Molecular and Cellular Endocrinology 235 (2005) 21–29

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Selective activation of PPARγ in breast, colon, and lung cancer cell lines

Clinton D. Allred, Michael W. Kilgore *

Department of Molecular and Biomedical Pharmacology, Markey Cancer Center, University of Kentucky College of Medicine, MS 305, Chandler Medical Center, 800 Rose St., Lexington, KY 40536, USA

Received 16 December 2004; received in revised form 16 February 2005; accepted 17 February 2005

Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) plays a critical albeit poorly defined role in the development and progression of several cancer types including those of the breast, colon, and lung. A PPAR response element (PPRE) reporter assay was utilized to evaluate the selective transactivation of PPARγ in 10 different cell lines including normal mammary epithelial, breast, lung, and colon cancer cells. Cells were treated with one of four compounds including rosglitizone (Ros), ciglitizone (Cig), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), or GW 9662 (GW). We observed differences in transactivation between cell lines from different tissue origin, across cell lines from a single tissue type, and selective modulation of PPARγ within a single cell line by different ligands. Interestingly, GW, a PPARγ antagonist in adipocytes, enhanced PPRE reporter activation in normal mammary epithelial cells while it had virtually no effect in any of the cancer cell lines tested. Within each cancer type, individual cell lines were found to respond differently to distinct PPARγ ligands. For instance, Ros, Cig, and PGJ_2 were all potent agonist of PPARγ transactivation in lung adenocarcinoma cell lines while these same ligands had no effect in squamous cell or large cell carcinomas of the lung.

Message levels of PPARγ and retinoid X receptor alpha (RXRα) in the individual cell lines were quantitated by real time-polymerase chain reaction (RT-PCR). The ratio of PPARγ to RXRα was predictive of how cells responded to co-treatment of Ros and 9-*cis*-retinoic acid, an RXRα agonist, in two out of three cell lines tested. These data indicate that PPARγ can be selectively modulated and suggests that it may be used as a therapeutic target for individual tumors.

Keywords: PPAR; Thiazoladinediones; Breast cancer; Colon cancer; Lung cancer

1. Introduction

The American Cancer Society estimated that collectively cancers of the breast, colon and lung accounted for 42% of all cancer deaths in men and 50% of all cancer deaths in women in 2004. In fact, breast, lung, and colon cancer rank as the top three types of malignancies identified in women today and one out of every eight women will develop breast cancer. In men, lung cancer is the most prevalent cause of cancer related death with malignancies of the prostate and colon following as next most common. A wide variety of chemotherapeutic options are being explored to treat these diseases. Novel therapeutic targets are being developed in an effort to identify endogenous, hormonal targets to either suppress cancer cell growth or induce apoptosis. One of the emerging targets for such treatments is peroxisome proliferator-activated receptor gamma (PPARγ).

The orphan nuclear receptor, PPARγ, is one of three of a family of receptors (PPARα, β, and γ) (Dreyer et al., 1992; Issemann and Green, 1990; Kliewer et al., 1994). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (Braissant et al., 1996; Kilgore et al., 1997; Lemberger et al., 1996; Nagy et al., 1998; Tontonoz et al., 1994). Several ligands of PPARγ have been identified including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), linoleic acid, lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone (Cig) and rosiglitazone (Ros) (Forman et al., 1995; Kliewer et al., 1997; Larsen

* Corresponding author. Tel.: +1 859 323 1821; fax: +1 859 323 1981.
E-mail address: M.Kilgore@uky.edu (M.W. Kilgore).

0303-7207/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2005.02.003

et al., 2003; McIntyre et al., 2003; Thoennes et al., 2000). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXRα), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (Kliewer et al., 1992; Tontonoz et al., 1994).

Recent evidence demonstrates that PPARγ is overexpressed in many different tumor types (DuBois et al., 1998; Tontonoz et al., 1997).In the breast, adenocarcinoma cells from patients expressed higher levels of PPARγ than normal epithelial cells from the surrounding mammary gland (Elstner et al., 1998). Similarly, in the colon, expression of PPARγ protein is significantly higher in human colon cancer sections when compared with non-tumor tissue (Chen et al., 2002). PPARγ has also been identified in both adenocaricoma and squamous cell carcinomas of the lung (Theocharis et al., 2002). Exposing cancer cells to PPARγ ligands produces physiological effects that may be exploited for treatment purposes. In culture, synthetic PPARγ ligands have been shown to inhibit growth of several tumor cell lines (Brockman et al., 1998; Elstner et al., 1998; Mueller et al., 1998). A number of studies have determined that PPARγ ligands induce cellular differentiation and/or apoptosis in breast, colon, and lung cancer cells (Chang and Szabo, 2002; Elstner et al., 1998; Mueller et al., 1998; Sarraf et al., 1998). The combination of receptor overexpression in tumors and known physiological effects of its ligands on cancer cells makes PPARγ a viable target of future chemotherapeutic agents.

The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (Fisher et al., 1998; Jordan and Morrow, 1999; Levenson and Jordan, 1999). It has been proposed that individual ligands may be able to act as selective PPARγ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (Sporn et al., 2001). We previously demonstrated that individual fatty acids can selectively activate a PPRE-reporter assay in estrogen-dependent breast cancer (MCF-7) cells (Thoennes et al., 2000). Specifically, omega-3 fatty acids inhibited transactivation of PPARγ to levels below control while omega-6, monounsaturated and saturated fatty acids stimulated the activity of the PPRE reporter. These data demonstrated that individual compounds can selectively activate PPARγ within the context of a single breast cancer cell line. However, compounds have yet to be identified that act as PPARγ agonists in one tissue while functioning as antagonists of the receptor in other tissues.

In the studies presented here, we sought to determine if distinct ligands could selectively activate PPARγ across different cell lines of mammary, colon, and lung origin. To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. Data from these experiments demonstrated that selective activation of PPARγ occurs in multiple ways. Distinct ligands selectively activate PPARγ dependent on the tissue type from which the cell line was derived. SPARM activity was also observed between different cell lines of the same tissue origin and individual ligands selectively activated the PPRE reporter within single cell lines. These data indicate that it may be possible to design PPARγ ligands that can be used to selectively mediate receptor activity and thus customize treatment regiments against specific cancers.

2. Materials and methods

2.1. Reagents

All PPARγ ligands were purchased from Cayman Chemical Company (Ann Arbor, MI). Ciglitazone (Cig) and GW9662 (GW) were solubilized in ethanol purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY). Rosiglitazone (Ros) was dissolved in dimethyl sulfoxide (DMSO) and PGJ_2 was solubilized in methyl acetate purchased from Sigma (St. Louis, MO).

2.2. Cells and cell culture

Ten individual cell lines were used in these experiments. Four mammary cell lines including normal mammary epithelial (HMEC) and three breast cancer cell lines (MCF-7, T47-D, and MDA-MB-231). Two colon cancer cell lines (Caco-2 and HT-29) and four lung cancer (A549, H358, H520, and H1299) were utilized. HMEC were purchased from Cambrex (Rockville, MD) while the MCF-7, T47-D, MDA-MB-231, H358, H520, and H1299 cells were all purchased from American Type Culture Collection (Bethesda, MD). The HT-29 and A549 cells were generously provided by Dr. David Kaetzel (University of Kentucky, College of Medicine) and the Caco-2 cells were a gift from Dr. Charlotte Kaetzel (University of Kentucky, College of Medicine). All cells were cultured in medium previously described to provide optimal conditions for their growth. When possible multiple cell lines were maintained in the same medium to reduce error when comparing across cell types. Cells were maintained in medium containing 10% FBS. All cell types were grown in medium lacking phenol red at 37 °C in a 5% CO_2 atmosphere. Cells were grown in T-75 flasks before being transferred to 12-well plates in preparation for transfection.

2.3. PPRE reporter plasmid

The reporter construct, 3XPPRE-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the mTK promoter between the *Xho*I and *Hin*dIII restriction enzyme sites of the pGL3 basic vector (Promega, Madison, WI). *Bam*HI and *Bgl*II were then used to release the 2.2 kb fragment containing the 3XPPRE-mTK-Luciferase. This fragment was ligated into the BamH I receptor site

of pRL-TK plasmid (Promega) completing the new reporter which contains both Luciferase and Renilla in a single expression plasmid. Renilla expression was used as a transfection efficiency control.

2.4. Transfection assays

Cells were transiently transfected with 5 μg of PPRE reporter plasmid per 12-well plate. Cells were transfected with ESCORT transfection reagent for 4 h. Cells were subsequently treated with either 10 μM Ros, 10 μM Cig, 1 μM PGJ_2, or 1 μM GW for 18 h. In all cases, PPARγ ligand concentrations for each compound used were those shown to be maximally effective following dose reponse studies (data not shown). Proper vehicle controls including ethanol, DMSO, and methyl acetate were run for each treatment group. Following treatment, cells were lysed in 50 μl passive lysis buffer and treated according to manufacturer's instructions (Promega dual luciferase assay kit). Luminometry was performed on a Berthold Lumat 9507 and data calculated as raw Luciferase Units (RLUs) divided by raw Renilla units. Mean fold induction was obtained by dividing the RLU data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of six in three separate experiments.

2.5. RNA preparation

Total RNA was isolated from cultured cells utilizing an RNeasy® Mini Kit (Qiagen, Valencia, CA) according to manufactures instructions. Untreated cells from each cell line were used for RNA isolation. All cell lines were maintained in optimal growth conditions prior to RNA collection. RNA was stored at −80 °C and concentration was measured at by spectrophotometry.

2.6. Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems (Foster City, CA) and used according to manufacturers instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to asses PPARγ (cat#: Hs00234592_m1) and RXRα (cat#: Hs00172565_m1) mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5′-nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a C_t cycle method.

At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number (C_t), which is inversely correlated to the abundance of the initial message level. C_t measures the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH (cat#: Hs99999905_m1), again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target C_t value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2^{-\Delta\Delta}C_t$, where $\Delta\Delta C_t$ represents ΔC_t, target sample minus ΔC_t, calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

2.7. Statistical analysis

As previously described (Thoennes et al., 2000), fold changes in luciferase to renilla ratios were subject to a two-factor analysis of variance (ANOVA) hypothesis testing ($\alpha = 0.05$) based on the two nominal variables of treatment and experimental date using a custom designed program running on the StatServer 6.1 (Insightful, Seattle, WA) server housed in the University of Kentucky's Department of Statistics. In every case, the post-hoc test, Tukey's pair-wise comparison, was performed to identify significant differences between the various treatments within a cell line. Briefly, the Tukey methodology simultaneously determined the presence of significant differences between individual treatment mean estimations across the entire balanced set of pairwise comparisons using the studentized range distribution, q. Mean fold changes in luciferase/renilla ratios of treatments compared to vehicle controls were displayed by column graph with one-half of the critical value for comparison from the Tukey's comparison as an estimation of error. Significant differences within those comparisons for a single cell line are designated by an alpha-numeric system.

For RT-PCR analysis, cycle threshold measurements, C_t, for the mRNA targets of both PPARγ and RXRα were repeated in triplicate within each cell line. The average C_t value for both PPARγ and RXRα in all 10 cell lines were subjected to analysis of variance hypothesis testing (ANOVA) using Microsoft Excel v10.0 at $\alpha = 0.05$ significance threshold. Following ANOVA, Fisher's least significant difference, LSD, pair-wise comparison was implemented post-hoc. Briefly, the LSD test determines a single critical value based on the mean squared error within groups and a critical value ($\alpha = 0.05$) found in the t distribution. If the average absolute difference between any two groups was greater than the LSD critical value, then the pair-wise comparison for those two groups were found to be significantly different at ($p < 0.05$).

3. Results

3.1. Effect of PPARγ ligands on reporter activation in breast cancer cells

Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were

treated with either vehicle control or PPARγ ligands for 18 h. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and PGJ_2 both significantly increased reporter activity over control (Fig. 1A). Interestingly, GW, a known antagonist of PPARγ, also significantly stimulated reporter activity. GW treatment did not change reporter activity compared to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1A). In MDA-MB-231 cells Ros, Cig, and PGJ_2 all significantly enhanced PPARγ activation over control, while these same three treatments also increased reporter activation in MCF-7 cells when compared to control (Fig. 1B). Both Ros and PGJ_2 treatments resulted in significantly higher activity than Cig in MCF-7 cells.

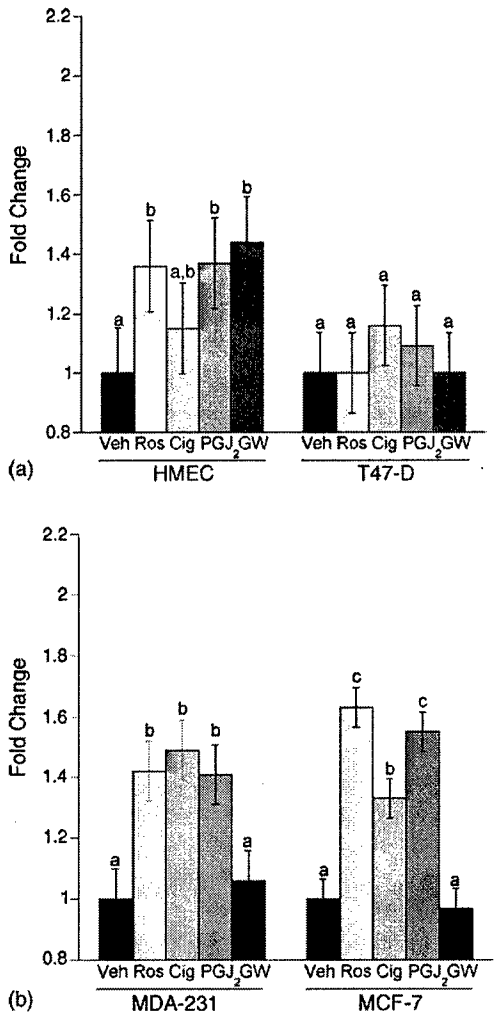


Fig. 1. (A and B) Effect of PPARγ ligands on reporter activation in breast cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

3.2. *Effect of PPARγ ligands on reporter activation in colon cancer cells*

Two colon cancer cell lines (HT-29 and Caco-2) were also tested in the same manner described for the mammary cells (Fig. 2). In general, HT-29 cells were more responsive to the PPARγ agonist than the Caco-2 cells. In the Caco-2 cells, only the Ros treatment caused significant increases in PPRE reporter activity when compared to control. Alternatively, in HT-29 cells Ros, Cig, and PGJ_2 treatments all resulted in significantly higher reporter activation when compared to vehicle control whereas, GW treatment was not significantly different from control in either colon cell line.

3.3. *Effect of PPARγ ligands on reporter activation in lung cancer cells*

To examine PPARγ activation in lung cancer cells, four cell lines were chosen. A549 and H358 cells were derived from adenocarcinoma lung tumors while H520 and H1299 cell lines are non-adenocarcinoma derived cell lines. H520 cells are lung squamous carcinoma cells and H1299 cells

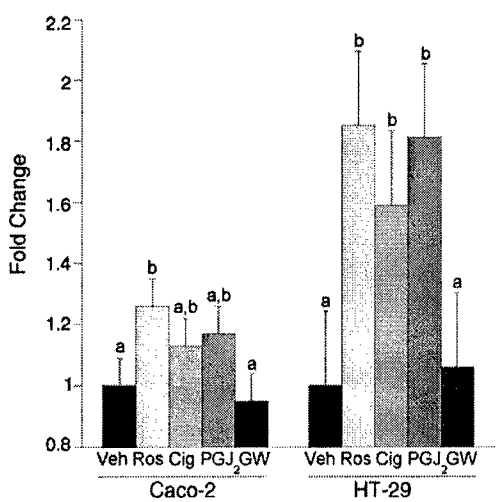


Fig. 2. Effect of PPARγ ligands on reporter activation in colon cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

were derived from a metastatic site of a patient with large cell carcinoma of the lung. Cells were transfected and treated in the same manner as the other cancer cell lines. Similar to the breast and colon differences in reporter activity were observed for individual cell lines. In general, non-adenocarcinoma (H520 and H1299) cells did not respond to PPARγ ligands as well as the adenocarcinoma (H358 and A549) cell lines (Fig. 3A and B). Also, as observed in various other cancer cell lines, significant differences between the relative activation of the reporter were seen with individual ligands within single cell lines. In H1299 cells, treatment with Cig, PGJ_2, or GW resulted in significantly higher activation of the PPRE reporter when compared to vehicle control while Ros and Cig treatment caused significant increases in activity in H520 cells (Fig. 3A). In H358 cells, Ros, Cig, and PGJ_2 all resulted in increased activation when compared to control (Fig. 3B). However, Ros treatment resulted in significantly greater reporter activity when compared to both Cig and PGJ_2 as well. Exposure of A549 cells to Ros, Cig, or PGJ_2 also caused a significant increase in activation of the PPRE reporter when compared to control (Fig. 3B). However, in these cells reporter activation was greatest in Cig treated cells and the fold change for this treatment was significantly greater than that in PGJ_2 treated cells. GW9662 treatment was not significantly different from control in H520, H358, or A549 cells.

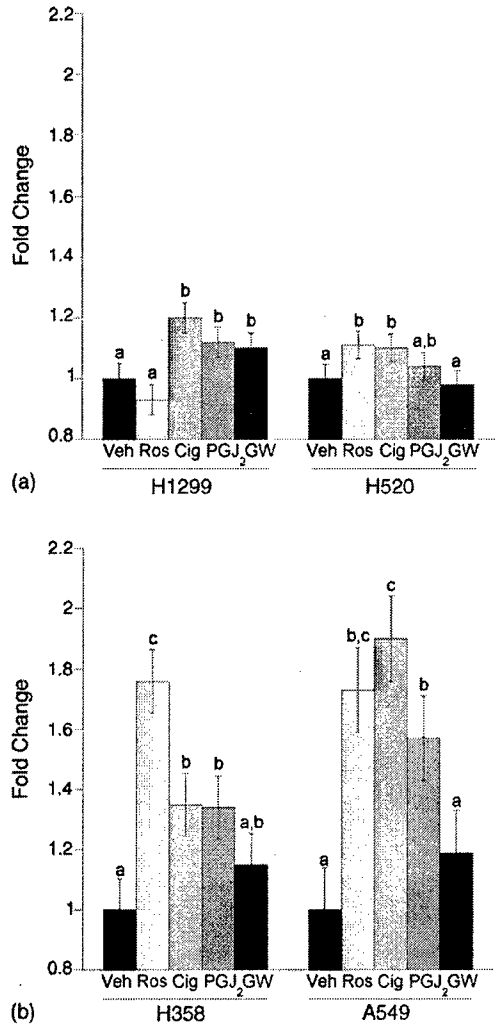


Fig. 3. (A and B) Effect of PPARγ ligands on reporter activation in lung cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

3.4. Expression of PPARγ and RXRα mRNA

mRNA levels of PPARγ and RXRα were measured in all cell lines (Fig. 4). Total RNA was isolated from untreated cells. H1299 had the lowest expression of both PPARγ and RXRα when compared to all other cells. H520 cells had the second lowest levels of PPARγ and RXRα, while HMEC and A549 cells were next highest. H358 cells had similar expression of PPARγ as HMEC and A549 cells, but had significantly more RXRα expression when compared to the same cell lines. T47-D cells had significantly lower levels of PPARγ than all of the cell lines except the H520 and H1299 cells. However, these cells had the highest expression of RXRα among all cell lines. Caco-2 cells expressed the second largest amount of PPARγ mRNA and had high RXRα levels with only MCF-7 and T47-D cells expressing more. HT-29 cells had higher mRNA levels of PPARγ when compared to all other cell lines and RXRα expression similar to Caco-2 and H358 cells. MCF-7 cells express significantly higher levels of PPARγ expression than all but four cell lines and higher RXRα than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPARγ mRNA levels similar to MCF-7 cells, but had lower RXRα expression with only two cell lines having significantly lower levels.

3.5. Effect of Ros and 9-cis-retinoic acid co-treatment on reporter activation in selected cell lines

Three cells lines were selected to determine if the relative expression of PPARγ and RXRα are predictive of the effect that co-treatment with a PPARγ agonist (Ros) and RXRα (9-*cis*-retinoic acid) agonist have on the PPRE reporter assay. HT-29, MCF-7, and MDA-MB-231 cells were selected to test this principal because Ros was shown to activate the reporter and the cell lines expressed varying levels of PPARγ to RXRα. MCF-7 cells were found to express more RXRα than PPARγ. Conversely, MDA-MB-231 and HT-29 cells expressed more PPARγ than RXRα. These three cell lines were transfected with the PPRE reporter construct and treated with

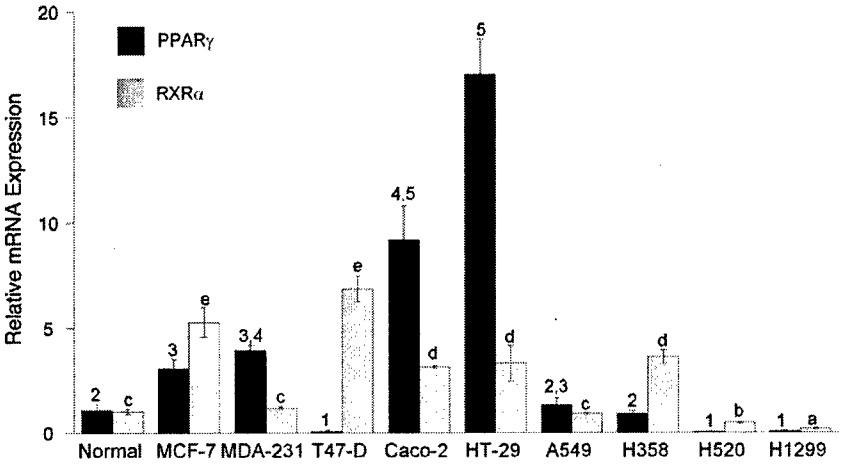


Fig. 4. Relative expression of PPARγ and RXRα cancer cell lines of the breast, colon, and lung. PPARγ and RXRα were detected by real-time PCR. Total mRNA was collected from untreated cells for each of the 10 cell lines. The relative expression levels of PPARγ and RXRα as compared to the endogenous control, human GAPDH were normalized to the expression of the targets in the normal mammary epithelia. Error bars represent the standard error of the mean.

either vehicle, Ros alone, or co-treated with Ros and 9-*cis*-retinoic acid. Ros alone and the co-treatment resulted in significantly higher activation of the reporter in all three cell lines when compared to vehicle controls (Fig. 5). In HT-29 cells, activation of the reporter was higher in the cells treated with Ros alone compared to those receiving the co-treatment. Conversely, co-treatment resulted in significantly greater reporter activity in the MCF-7 and MDA-MB-231 cells when compared to Ros treatment alone.

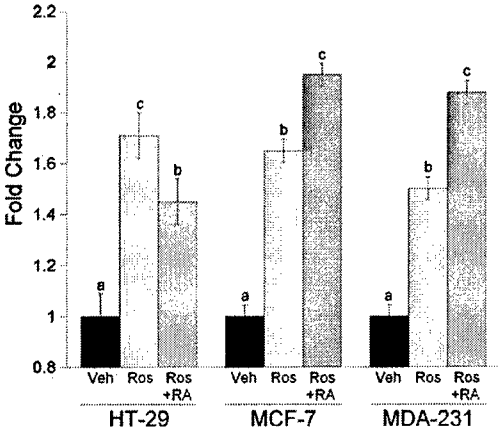


Fig. 5. Effect of cotreatment with Rosiglitazone and 9-*cis*-retinoic acid on reporter activation in various. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with either rosiglitazone (Ros) or rosiglitazone plus 9-*cis*-retinoic acid (Ros + RA) for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

4. Discussion

Data from the present study demonstrate that individual PPARγ ligands have the ability to selectively activate a PPRE reporter in cancers of the breast, colon, and lung. Differences in PPRE reporter activation were observed between cells derived from different tissue types as well as between cell lines of the same cancer type. Also, within a single cell line, individual ligands selectively induced PPRE reporter activity. Expression of PPARγ and RXRα mRNA were measured in all cell lines in the absence of treatment, but expression was not predictive of how individual cell lines responded to ligand treatment. Finally, differences in how individual cell lines responded to co-treatment with PPARγ and RXRα agonists were observed.

A growing body of evidence indicates PPARγ is involved in both breast cancer development and progression. PPARγ(+/−) mice had almost three-fold increased incidence of mammary adenocarcinomas and decreased survival rate when compared to PPARγ(+/+) litermates (Nicol et al., 2004). Several reports have demonstrated that treating animals with PPARγ ligands prior to chemical induction of mammary tumors is protective against tumor development (Mehta et al., 2000; Suh et al., 1999). It appears that expression and transactivation of PPARγ is protective against breast tumor formation particularly when activated by PPARγ ligands. Our data demonstrate that some ligands are more effective than others in transactivating PPARγ in normal mammary epithelia.

Once a breast tumor has formed, PPARγ appears to have multiple effects. In vitro, treatment of breast cancer cells with troglitazone results in lipid accumulation, changes in gene expression associated with cellular differentiation, reduction in growth rate and clonogenic capacity (Mueller et al., 1998). Others have observed that distinct PPARγ ligands induce apoptosis (Elstner et al., 1998). Conversely, a recent

report by Saez et al. (2004) found that when mice expressing a constitutively active form of PPARγ in the mammary gland were crossed with mice prone to mammary gland cancer, bigenic animals develop tumors that express higher levels of markers of malignancy. The authors conclude that once an initiating event takes place, increased PPARγ signaling serves as a tumor promoter in the mammary gland of these experimental animals. Collectively, these data suggest that the physiological consequence of PPARγ activation is dependent on many factors including the stage of development of the specific breast cancer cell. Our demonstration that individual PPARγ ligands distinctively modulate PPRE reporter activity in breast cancer cell lines differently has implications for breast cancer treatment. Specifically, T47-D cells were fairly unresponsive to any of the three PPARγ agonists tested, whereas, Ros, Cig, and PGJ_2 significantly increased reporter activity in MCF-7 and MDA-MB-231 cells. It can be concluded that individual breast cancer cell types are likely to respond to PPARγ ligands in unique physiological ways and our data suggests that, in part, variant cellular responses are the result of selective PPARγ transactivation.

PPARγ also influences colon tumor development and growth. In mice predisposed to the development of intestinal polyps caused by a mutation in the adenomatous polyposis coli (APC) gene, treatment with troglitazone or Ros increases both the number and size of intestinal polyps (Lefebvre et al., 1998; Saez et al., 1998). These data are partially explained by studies demonstrating that PPARγ looses its ability to influence colon tumorgenesis in mice with a mutated APC gene, where as in wild-type APC mice, PPARγ functions as a tumor suppressor (Girnun et al., 2002). Conversely, PPARγ ligands reduce aberrant crypt foci (ACF) formation in mice following tumor induction by azoxymethane (Osawa et al., 2003). Differences in the effects of PPARγ ligands in these two models demonstrates that like mammary cells, colon cancer cells respond to a single PPARγ ligand differently dependent on the cell characteristics. When we examined the ability of PPARγ ligands to activate the PPRE reporter construct in two colon cancer cell lines, differences in cellular responsiveness was revealed. Ros significantly increased reporter activity in both Caco-2 and HT-29 cells though the level of responsiveness was much greater in the HT-29 cells. Furthermore, Cig and PGJ_2 did not significantly enhance reporter signal in Caco-2 cells, but were strong agonists in the HT-29 cells. Selective modulation of PPARγ transactivation can explain the variant physiological responses observed in different colon cancer animal models. Differences in ligand activity could have significant impact on colon cancer treatment strategy as a number of studies have shown that PPARγ ligands affect colon tumor cell progression. Treatment of colon cancer cells with PPARγ agonists inhibits their growth in vivo (Brockman et al., 1998; Kitamura et al., 1999; Sarraf et al., 1998; Shimada et al., 2002) and in vitro (Sarraf et al., 1998). Inhibition of growth is often attributed to PPAR induced apoptosis and DNA fragmentation (Chen et al., 2002; Shimada et al., 2002; Yang and Frucht, 2001).

In the lung, epithelial cells possess 15-lipoxygenases which produce a variety of metabolic products including 15(S)-hydroxyeicosatetranoic acid (15(S)-HETE) (Profita et al., 2000). In A549 cells, 15(S)-HETE has been demonstrated to induce apoptosis by binding to PPARγ (Shankaranarayanan and Nigam, 2003). Similarly, treatment of adenocarcinoma (A549) cells with Cig resulted in growth inhibition (Chang and Szabo, 2002); however, this inhibition was not observed in either squamous cell carcinoma (H520) or large cell carcinoma (H1299) cell types (Chang and Szabo, 2002). In the present study, two adenocarcinoma (A549 and H358) and two nonadenocarcinoma (H520 and H1299) cell lines were selected for evaluation. The nonadenocarcinoma cell lines were highly unresponsive to the PPARγ ligands when compared to the adenocarcinoma cells. Ros and Cig significantly increased reporter activity in H1299 and H520 cells, but fold change compared to control was relatively small in these cells. Conversely, A549 and H358 cells were highly responsive to the PPARγ agonists. These data suggest that the varying effects of Cig on adenocarcinoma versus nonadenocarcinoma cells observed in Chang et al., 2002 are likely the down stream result of selective PPRE transactivation.

One focus of these studies was to determine whether individual ligands of PPARγ could act as SPARMs. We present evidence indicating that within each tissue type, individual ligands are capable of selectively activating the PPRE reporter construct dependent on the individual cell line tested. However, individual ligands had unique effects across tissue types as well. For instance, we report that GW, a known PPARγ antagonist in adipocytes (Leesnitzer et al., 2002; Starkey et al., 2003), significantly increased reporter activity in HMECs. This effect was observed in no other cell line except H1299 cells and in those cells the magnitude of change was very small leaving in question its biological significance. These findings are significant because they suggest that an individual compound can function as a PPARγ antagonist in one tissue and as an agonist in other tissues. It is possible that the agonist activity of GW is specific to normal epithelial cells and that changes occur during cancer cell formation that results in the loss of this responsiveness. It is also possible that the actions of GW are mammary specific. Further, investigation is necessary to explore these possibilities.

Another objective of these studies was to determine if PPARγ mRNA expression is predictive of a cell line's responsiveness to PPARγ ligands with regards to PPRE activation. For three of the cell lines (MCF-7, MDA-MB-231, and HT-29) increased relative expression of PPARγ over HMECs correlated with enhanced reporter activity when exposed to the PPARγ agonists. However, A549 and H358 had higher reporter activity in response to the individual PPARγ agonist compared to Caco-2 cells despite the fact that A549 and H358 cells express much lower levels of PPARγ than the Caco-2s. Therefore, PPARγ mRNA levels alone are not predictive of PPARγ mediated PPRE activation. These data led us to ex-

plore the possibility that variances of expression of RXRα, the heterodimic partner of PPARγ, and it's relation to the levels of PPARγ in the different cell lines may play a critical role in PPARγ's ability to activate the PPRE reporter construct. Here we demonstrate that the cell lines differed in their relative expression of PPARγ to RXRα; however, there was no unifying pattern of receptor expression that was predictive of ligand activity in the reporter assay.

Unique expression patterns of PPARγ relative to RXRα in certain cell lines led us to hypothesize that the relative expression of these receptors may be used to identify cell lines in which co-treatment with an RXRα agonist (9-*cis*-retinoic acid) would enhance PPRE reporter activation. Three cell lines were chosen to test this hypothesis. HT-29 and MDA-MB-231 cells express higher levels of PPARγ relative to RXRα and would therefore, not be expected to have increased reporter activity with co-treatment (Ros + 9-*cis*-retinoic acid) compared to Ros alone. Conversely, MCF-7 cells having higher expression of RXRα relative to PPARγ would supposedly demonstrate enhanced reporter activity when treated with both ligands compared to Ros alone. HT-29 cells showed no additional reporter activity in cells co-treated with Ros and 9-*cis*-retinoic acid over those treated with Ros alone. MCF-7 cells had enhanced activation of the PPRE reporter with co-treatment over Ros treatment alone. These two cell lines support the possibility that relative expression levels may be predictive in identifying cells that will more readily respond to co-treatment with both ligands. However, MDA-MB-231 cells did not support this hypothesis. Co-treatment with both ligands had a significantly higher effect on reporter activity when compared to single treatment with Ros even though relative PPARγ expression was higher than RXRα expression in these cells. These data suggest that while this approach may work for certain cancer cell types, receptor expression alone may not be predictive for how cells will respond to co-treatment. A better understanding of how PPARγ and RXRα function in each of the cell lines will be necessary before the predictive value of receptor expression can be realized.

In conclusion, PPARγ ligands, have distinct activities within a cell type, between tumor cells derived from the same tissue, and across distinct tissues. Although we used only one type of PPRE reporter construct in these experiments, the selective modulation of PPARγ within individual cells is likely to be a gene/promoter specific event. Nonetheless, utilizing this reporter of PPAR transactivation, differences were observed in all three cancer types (breast, colon, and lung) where a single ligand enhanced reporter activation in certain cell types, but had minimal to no effect in other cell lines of the same malignancy type. Finally, our data demonstrates that individual PPARγ ligands can selectively activate reporter activity within a single cancer cell type. These data suggest that the effectiveness of PPARγ as a target for chemotherapeutic treatment will greatly depend on the cell that is treated which opens the possibility of utilizing PPARγ for targeted gene therapy.

Acknowledgments

This work was supported by grants DAMD17-97-1-7248 from the US Department of Defense, R01-CA 95609-1, 5-K12-DA-14040-02 and NCRR-P20-RR15592 from the NIH to MWK, and W81XWH-04-1-0532 from the Department of Defense to CDA. We would like to thank Dr. Charlotte Kaetzel and Dr. David Kaetzel for providing cell lines used in these studies. We also thank Dr. Scott Diamond for his insight into the preparation of this manuscript and Dr. Arnold Stromberg for statistical support.

References

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., Wahli, W., 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 137, 354–366.

Brockman, J.A., Gupta, R.A., Dubois, R.N., 1998. Activation of PPARgamma leads to inhibition of anchorage-independent growth of human colorectal cancer cells. Gastroenterology 115, 1049–1055.

Chang, T.H., Szabo, E., 2002. Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-gamma and inhibitors of histone deacetylase in adenocarcinoma of the lung. Clin. Cancer Res. 8, 1206–1212.

Chen, G.G., Lee, J.F., Wang, S.H., Chan, U.P., Ip, P.C., Lau, W.Y., 2002. Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer. Life Sci. 70, 2631–2646.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., Wahli, W., 1992. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68, 879–887.

DuBois, R.N., Gupta, R., Brockman, J., Reddy, B.S., Krakow, S.L., Lazar, M.A., 1998. The nuclear eicosanoid receptor, PPARgamma, is aberrantly expressed in colonic cancers. Carcinogenesis 19, 49–53.

Elstner, E., Muller, C., Koshizuka, K., Williamson, E.A., Park, D., Asou, H., Shintaku, P., Said, J.W., Heber, D., Koeffler, H.P., 1998. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc. Natl. Acad. Sci. U.S.A. 95, 8806–8811.

Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., Cronin, W.M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L., Wolmark, N., 1998. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J. Natl. Cancer Inst. 90, 1371–1388.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M., 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83, 803–812.

Girnun, G.D., Smith, W.M., Drori, S., Sarraf, P., Mueller, E., Eng, C., Nambiar, P., Rosenberg, D.W., Bronson, R.T., Edelmann, W., Kucherlapati, R., Gonzalez, F.J., Spiegelman, B.M., 2002. APC-dependent suppression of colon carcinogenesis by PPARgamma. Proc. Natl. Acad. Sci. U.S.A. 99, 13771–13776.

Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347, 645–650.

Jordan, V.C., Morrow, M., 1999. Tamoxifen, raloxifene, and the prevention of breast cancer. Endocrinol. Rev. 20, 253–278.

Kilgore, M.W., Tate, P.L., Rai, S., Sengoku, E., Price, T.M., 1997. MCF-7 and T47D human breast cancer cells contain a functional peroxisomal response. Mol. Cell. Endocrinol. 129, 229–235.

Kitamura, S., Miyazaki, Y., Shinomura, Y., Kondo, S., Kanayama, S., Matsuzawa, Y., 1999. Peroxisome proliferator-activated receptor gamma induces growth arrest and differentiation markers of human colon cancer cells. Jpn. J. Cancer Res. 90, 75–80.

Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K., Evans, R.M., 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. U.S.A. 91, 7355–7359.

Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W., Willson, T.M., Lenhard, J.M., Lehmann, J.M., 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc. Natl. Acad. Sci. U.S.A. 94, 4318–4323.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., Evans, R.M., 1992. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358, 771–774.

Larsen, T.M., Toubro, S., Astrup, A., 2003. PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy? Int. J. Obes. Relat. Metab. Disord. 27, 147–161.

Leesnitzer, L.M., Parks, D.J., Bledsoe, R.K., Cobb, J.E., Collins, J.L., Consler, T.G., Davis, R.G., Hull-Ryde, E.A., Lenhard, J.M., Patel, L., Plunket, K.D., Shenk, J.L., Stimmel, J.B., Therapontos, C., Willson, T.M., Blanchard, S.G., 2002. Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662. Biochemistry 41, 6640–6650.

Lefebvre, A.M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J.C., Geboes, K., Briggs, M., Heyman, R., Auwerx, J., 1998. Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. Natl. Med. 4, 1053–1057.

Lemberger, T., Braissant, O., Juge-Aubry, C., Keller, H., Saladin, R., Staels, B., Auwerx, J., Burger, A.G., Meier, C.A., Wahli, W., 1996. PPAR tissue distribution and interactions with other hormone-signaling pathways. Ann. N. Y. Acad. Sci. 804, 231–251.

Levenson, A.S., Jordan, V.C., 1999. Selective oestrogen receptor modulation: molecular pharmacology for the millennium. Eur. J. Cancer. 35, 1974–1985.

McIntyre, T.M., Pontsler, A.V., Silva, A.R., St Hilaire, A., Xu, Y., Hinshaw, J.C., Zimmerman, G.A., Hama, K., Aoki, J., Arai, H., Prestwich, G.D., 2003. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. Proc. Natl. Acad. Sci. U.S.A. 100, 131–136.

Mehta, R.G., Williamson, E., Patel, M.K., Koeffler, H.P., 2000. A ligand of peroxisome proliferator-activated receptor gamma, retinoids, and prevention of preneoplastic mammary lesions. J. Natl. Cancer Inst. 92, 418–423.

Mueller, E., Sarraf, P., Tontonoz, P., Evans, R.M., Martin, K.J., Zhang, M., Fletcher, C., Singer, S., Spiegelman, B.M., 1998. Terminal differentiation of human breast cancer through PPAR gamma. Mol. Cell 1, 465–470.

Nagy, L., Tontonoz, P., Alvarez, J.G., Chen, H., Evans, R.M., 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 93, 229–240.

Nicol, C.J., Yoon, M., Ward, J.M., Yamashida, M., Fukamachi, K., Peters, J.M., Gonzalez, F.J., 2004. PPAR{gamma} influences susceptibility to DMBA-induced mammary, ovarian and skin carcinogenesis. Carcinogenesis 25, 1747–1755.

Osawa, E., Nakajima, A., Wada, K., Ishimine, S., Fujisawa, N., Kawamori, T., Matsuhashi, N., Kadowaki, T., Ochiai, M., Sekihara, H., Nakagama, H., 2003. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. Gastroenterology 124, 361–367.

Profita, M., Sala, A., Riccobono, L., Pace, E., Paterno, A., Zarini, S., Siena, L., Mirabella, A., Bonsignore, G., Vignola, A.M., 2000. 15(S)-HETE modulates LTB(4) production and neutrophil chemotaxis in chronic bronchitis. Am. J. Physiol. Cell. Physiol. 279, C1249–C1258.

Saez, E., Rosenfeld, J., Livolsi, A., Olson, P., Lombardo, E., Nelson, M., Banayo, E., Cardiff, R.D., Izpisua-Belmonte, J.C., Evans, R.M., 2004. PPAR gamma signaling exacerbates mammary gland tumor development. Genes Dev. 18, 528–540.

Saez, E., Tontonoz, P., Nelson, M.C., Alvarez, J.G., Ming, U.T., Baird, S.M., Thomazy, V.A., Evans, R.M., 1998. Activators of the nuclear receptor PPARgamma enhance colon polyp formation. Natl. Med. 4, 1058–1061.

Sarraf, P., Mueller, E., Jones, D., King, F.J., DeAngelo, D.J., Partridge, J.B., Holden, S.A., Chen, L.B., Singer, S., Fletcher, C., Spiegelman, B.M., 1998. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. Natl. Med. 4, 1046–1052.

Shankaranarayanan, P., Nigam, S., 2003. IL-4 induces apoptosis in A549 lung adenocarcinoma cells: evidence for the pivotal role of 15-hydroxyeicosatetraenoic acid binding to activated peroxisome proliferator-activated receptor gamma transcription factor. J. Immunol. 170, 887–894.

Shimada, T., Kojima, K., Yoshiura, K., Hiraishi, H., Terano, A., 2002. Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. Gut 50, 658–664.

Sporn, M.B., Suh, N., Mangelsdorf, D.J., 2001. Prospects for prevention and treatment of cancer with selective PPARgamma modulators (SPARMs). Trends Mol. Med. 7, 395–400.

Starkey, K., Heufelder, A., Baker, G., Joba, W., Evans, M., Davies, S., Ludgate, M., 2003. Peroxisome proliferator-activated receptor-gamma in thyroid eye disease: contraindication for thiazolidinedione use? J. Clin. Endocrinol. Metab. 88, 55–59.

Suh, N., Wang, Y., Williams, C.R., Risingsong, R., Gilmer, T., Willson, T.M., Sporn, M.B., 1999. A new ligand for the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), GW7845, inhibits rat mammary carcinogenesis. Cancer Res. 59, 5671–5673.

Theocharis, S., Kanelli, H., Politi, E., Margeli, A., Karkandaris, C., Philippides, T., Koutselinis, A., 2002. Expression of peroxisome proliferator activated receptor-gamma in non-small cell lung carcinoma: correlation with histological type and grade. Lung Cancer 36, 249–255.

Thoennes, S.R., Tate, P.L., Price, T.M., Kilgore, M.W., 2000. Differential transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells. Mol. Cell. Endocrinol. 160, 67–73.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., Spiegelman, B.M., 1994. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8, 1224–1234.

Tontonoz, P., Singer, S., Forman, B.M., Sarraf, P., Fletcher, J.A., Fletcher, C.D., Brun, R.P., Mueller, E., Altiok, S., Oppenheim, H., Evans, R.M., Spiegelman, B.M., 1997. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. Proc. Natl. Acad. Sci. U.S.A. 94, 237–241.

Yang, W.L., Frucht, H., 2001. Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. Carcinogenesis 22, 1379–1383.